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EFFECT OF PURIFIED FIBRINOGENASE FROM THE VENOM OF THE CHINESE HABU SNAKE (*Trimeresurus mucrosquamatus*) ON FIBRINOGEN AND THROMBIN

Zhang Yun Xiong Yuliang

(Kunming Institute of Zoology, Academia Sinica)

Key words: Fibrinogen degraded products, Fibrinogen, Snake venom, Thrombin

Abstract

The fibrinogenase of *T. mucrosquamatus* venom (TMVFg) could hydrolyze the tripeptide Bz-Phe-Val-Arg-PNA, but it showed low enzymatic activity toward Cbz-Gly-Pro-Arg-PNA which was a good substrate of thrombin. TMVFg apparently prolonged plasma thrombin time, plasma recalcification time and fibrinogen solution thrombin time. By using MonoQ ion-exchange chromatography (EPLC), fibrinogen products degraded with TMVFg were isolated. One, designated as FDP Ⅰ, inhibited fibrin clot formation, Another one, designated as FDP Ⅱ, not only enhanced the fibrin clot but also inhibited platelet aggregation induced by TMVA (a platelet aggregation inducer isolated from the venom of *T. mucrosquamatus*) and this inhibitory effect was dose dependent. FDP Ⅱ had no effect on platelet aggregation induced by ADP or by arachidonic acid (AA). The results also showed that TMVFg had certain inhibitory effect on thrombin activities.

INTRODUCTION

The biological activities of bibrinogen degraded products (FDP) have been extensively studied. They have special biological activities such as increasing capillary permeabilily, enhancing the releasing of prostaglandin (GERDIN *et al.*, 1980), inhibiting platelet aggregation induced by ADP (MCMANAMA *et al.*, 1986) and inhibiting fibrin polymerization (FURLAN *et al.*, 1983). These products are associated with the occurrence of some diseases. Up to now, the studies of snake venom proteinase are mainly concentrated on their biochemical properties. Obviously, it is important to investigate the effects of snake venom proteinase on blood coagulation factor such as fibrinogen and thrombin. We have isolated a fibrinogenase (TMVFg) from the venom of *T. mucrosquamatus* and studied their physico-chemical properties. It was of interest to study its effects on fibrinogen and thrombin.

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MATERIALS AND METHODS

The fibrinogenase of *Trimeresurus mucrosquamatus* venom (TMVFg) was prepared according to the method described in the previous paper. Human fibrinogen, human thrombin, ADP and arachidonic acid were purchased from Sigma Chemical Co. (St. Louis, U. S. A.). MonoQ column (FPLC, Model GP—250) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). TMVA (a platelet aggregation inducer from *T. mucrosquamatus* venom) was purified by our laboratory. Experimental rabbits were supplied by our institute. Other chemicals used were analytical grade and purchased from commercial sources.

The prolonging effects of TMVFg on plasma recalcification time, plasma thrombin time

The blood sample was collected from rabbit heart and mixed with 3.8% sodium citrate (9:1, v/v). The citrated blood was immediately centrifuged for 10 min at 120× g at room temperature to obtain platelet-rich plasma. Platelet-poor plasma was obtained by centrifugation of the citrated blood for 20 min at 1500× g at 4 ℃. For platelet aggregation, the turbidimetric method of WESTWICK *et al.* (1980) was used. Plasma thrombin time, plasma recalcification time were carried out as described by XU (1979). At first, preincubated different dosages of TMVFg (0.1 ml) with plasma (0.1 ml) at 37℃ for 5 min., then added throbmin (0.1 ml, 10 U/ml) or 0.1 ml 25 m*M* CaCl to record the clotting time.

Determination of fibrinogenolytic activity

Fibrinogenolytic activity was measured by the modified method of OUYANG and TENG (1976). An equal part of 0.5% (w/v) human fibrinogen solution in 25 m*M* Tris-HCl buffer, pH 8.0 were mixed and incubated with the purified enzyme (final concentration, 25 μg/ml) at 37℃ for various time intervals. 0.5 ml of the above incubation mixture was then withdrawn for the clottable protein assay. For determining the effect of TMVFg on fibrinogen solution thrombin time, human thrombin (10 U/ml, 0.1 ml) was added in the same samples (0.2 ml) to record the clotting time.

Measurement of fibrinogen-fibrin polymerization

The turbidimetric method of MOSESSON *et al.* (1967) was modified as follows, 3.0 ml of 0.5% (M/V) human fibrinogen in 10 m*M* Tris-HCl buffer, pH 7.6 was mixed and incubated with 0.4 ml of the purified enzyme (TMVFg, 100 μg) at 37℃ for various time intervals, then 0.1 ml human thrombin (10 U/ml) was added and rapidly mixed. The absorbances were monitored on Shimadzu spectrophotometer (Model UV-120-02, Japan) at 381.5 nm during fibrin polymerization.

Isolation of fibrinogen products degraded with TMVFg

1 ml of 0.5% (W/V) human fibrinogen in 5 m*M* Tris-HCl buffer, pH 8.0 was mixed and incubated with 0.3 ml TMVFg (75μg) at 37℃ for 1 hour. The mixture was centrifuged for 12 min at 1500× g. The supernatant was applied to the MonoQ column and eluted with a gradient of NaCl (A buffer, 25 m*M* Tris-HCl, pH 8.0; B buffer, 25 m*M* Tris-HCl, pH 8.0 containing 1 *M* NaCl) at a flow rate of 0.8ml/min.

For studying the effect of FDP I and I on fibrinogen-fibrin polymerization, the turbidimetric method mentioned above was used.

Determination of thrombin activity

Tripeptide Cbz-Gly-Pro-Arg-PNA was used as a substrate of thrombin. 0.2 ml human thrombin (10 U/ml) was mixed and incubated with 0.2 ml TMVFg (40 μg) at 37℃ for various time intervals. 4.2 ml of 25 m*M* Tris-HCl buffer, pH 8.0 and 0.4 ml of tripeptide substrate (1.0 mg/ml, W/V, in the buffer) were then added. The absorbances were measured on Shimadazu spectrophotometer at 405 nm.

The turbidimetric method mentioned above was also used to measure the thrombia activity. 0.2 ml human thrombin (10 U/ml) was mixed and incubated with 0.4 ml TMVFg (80μg) at 37℃ for various time intervals. Then 3.0 ml of 0.5% (W/V) human fibrinogen in 20 m*M* Tris-HCl buffer, pH 7.6 was added.

Another thrombin substrate Bz-Phe-Val-Arg-PNA was used to detect the TMVFg activity. 50μg of

TMVFg was incubated with the substrate at 37℃ for 15 min according to the method of SVENDSEN (1972).

RESULTS

Effects of TMVFg on thrombin time and recalcification time

After treatment of plasma with TMVFg, an apparent prolongation of recalcification time and thrombin time were observed (Fig. 1). These effects were depend on the dose of TMVFg. When a low dose was used, only a slight prolongation of recalification time appeared.

Effect of TMVFg on polymerization of fibrinogen

Fig. 2 showed the effect of TMVFg on thrombin time of human fibrinogen. When fibrinogen was preincubated with TMVFg for 5 min, prolongation of thrombin time reached maximum, then decreased with the increase in preincubation time, and then increased again after preincubation for more than 40 min. The contents of clottable protein were also determined at different preincubation times under the same conditions. When preincubated for 40 min, over 50% of the clottable protein was still remained. If the remaining fibrinogen was kept above 40% of the original the clot time of fibrinogen was not prolonged obviously. The same result was reported by RATNOFF *et al.*, (1978). Thus it was demonstrated that the prolongation of clot time was caused by the fibrinogen degraded products, not by the decreasing of fibrinogen content.

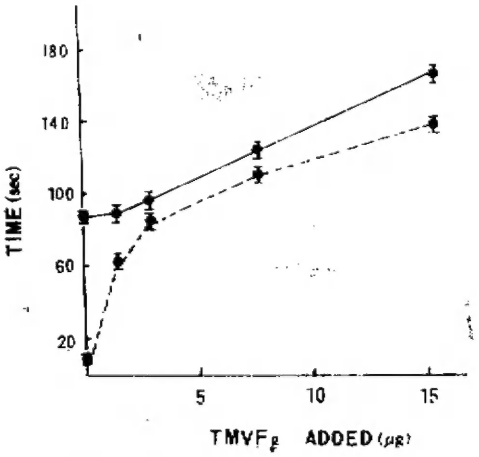

Fig. 1. The effects of TMVFg on plasma thrombin time, plasma recalcification time. (●—●) Plasma recalcification time, (●·····●) Plasma thrombin time. Means ± S. E. of four determinations were presented.

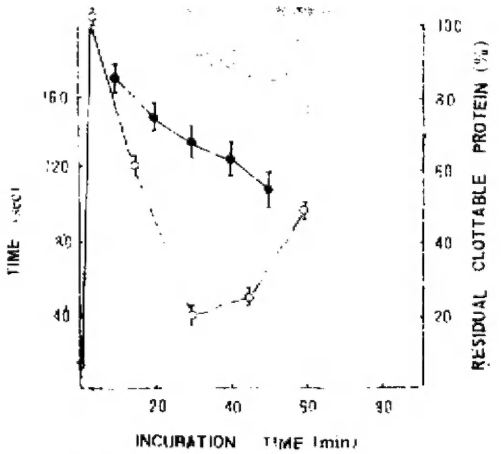

Fig. 2. The effects of TMVFg on human fibrinogen and fibrinogen solution thrombin time. (●—●), residual percentage of clottable protein. (○—○), human fibrinogen solution thrombin time. Means ± S. E. of triplicate experiments were presented.

Fibrinogen-fibrin polymerization

Using spectrophotometer to follow the process of fibrinogen-fibrin polymerization induced by thrombin after preincubation of human fibrinogen with TMVFg (Fig. 3), polymerization of fibrin was remarkably inhibited when preincubation time was 5 min. However, with the increase in preincubation time, both "lag phase" and "enhancing phase" of fibrinogen-fibrin polymerization appeared; the longer the preincubation time was, the more obvious both "phases" were. The results demonstrated that there were various kinds of fibrinogen degradation products degraded by TMVFg. The "early" degraded products possessed anti-polymerizing effect, whereas the "later" degraded products possessed anti-thrombin action and enhancing polymerization.

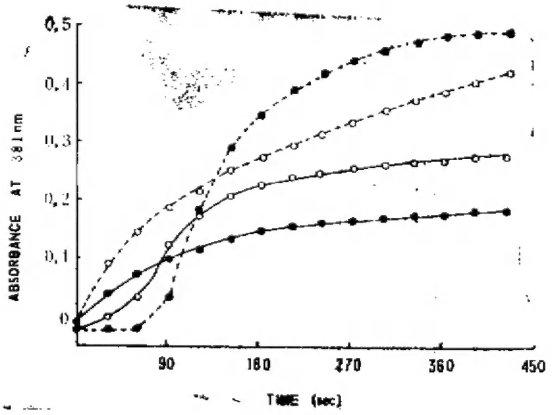

Fig. 3. Clotting of human fibrinogen by human thrombin at pH 7.6, ionic strengh 0.01 M, after preincubating the fibrinogen with TMVFg (100 μg) at 37℃ for various time intervals. Preincubatiom time were 5 min (●—●), 15 min (○—○), 90 min (●----●), control (○····○).

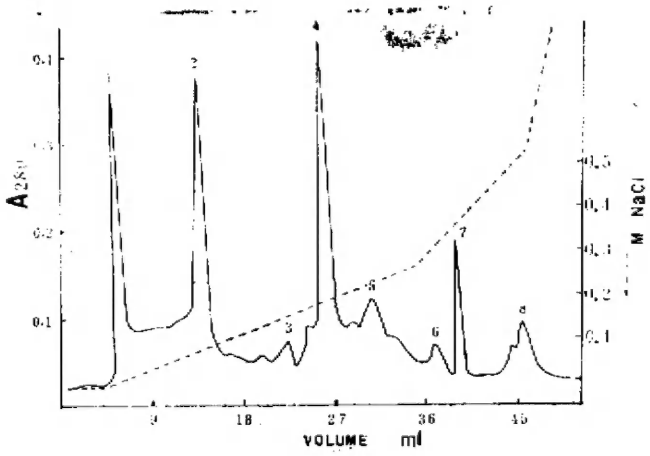

Fig. 4. FPLC isolation scheme of fibrinogen products degraded by TMVFg

Isolation of human fibrinogen degraded products

Human fibrinogen products degraded with TMVFg were isolated by FPLC. The Products (human fibrinogen was preincubated with TMVFg at 37℃ for 60 min) was separated into eight fractions (Fig. 4). The arginine esterase activity was present in fraction 6. By biological activity assay of each fraction, we found that fraction 3 (designated as FDP Ⅰ) had an inhibitory effect on formation of fibrin clot, whereas fraction 8 (designated as FDP Ⅱ) had an enhancing effect on the formation of fibrin clot (Fig. 5). It is very intersting to identify the physico-chemical properties of FDP Ⅰ and FDP Ⅱ.

Inhibitory effect of FDP Ⅰ on platelet aggregation

FDP Ⅰ inhibited platelet aggregation induced by TMVA, a platelet aggregation inducer isolated from *T. mucrosquamatus* venom, being different from those of ADP and arachidonic acid in inducing mechanism (WANG *et al.*, 1987). This inhibition was dose dependent (Fig. 6). FDP Ⅰ did not affect the platelet aggregation induced by ADP or by arachidonic acid.

Inhibitory effect of TMVFg on human thrombin

TMVFg showed a very low enzymatic activity on tripeptide Cbz-Gly-Pro-Arg-PNA which was a good thrombin substrate. When incubated TMVFg with human thrombin at 37℃ for various time intervals,

TMVFg showed an inhibitory effect on thrombtin activity (Fig. 7) and the inhibitory effect was dependent on preincubation time. The same results were obtained if fibrin clot method was used for thrombin activity determination.

TMVFg could hydrolyze tripeptide Bz-Phe-Val-Arg-PNA.

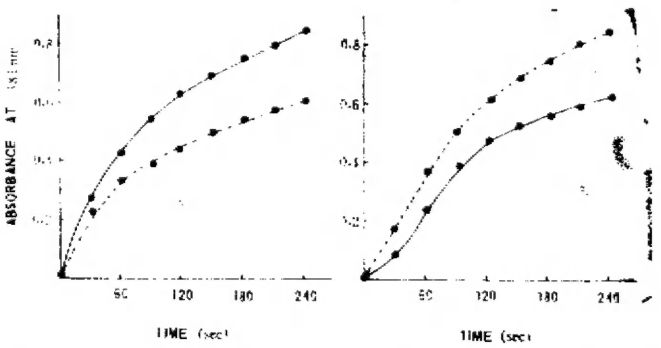

Fig. 5. Clotting of human fibrinogen by human thrombin in the presence and absence of FDP fraction. Right part, the effect of FDP Ⅰ on clotting of fibrinogen in 0.02*M* Tris buffer, pH 7.6, control(●-----●), with 0.4 ml of 5 μg FDP Ⅰ(●—●). Left part, the effect of FDP Ⅱ on clotting of fibrinogen in 0.05*M* Tris buffer, pH 7.6. control (●----●), with 0.4 ml of 7 μg. FDP Ⅱ (●—●).

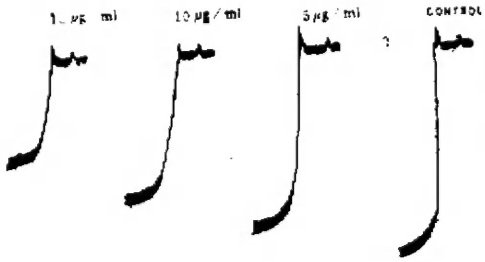

Fig. 6. Inhibitory effect of FDP Ⅱ produced by TMVFg on TMVA-induced platelet aggregation. FDP Ⅱ was added in platelet rich plasma (final concentration, 5, 10, 15 μg/ml). After 1 min stirring, TMVA (76 μg/ml, final concentration) was added to trigger the aggregation.

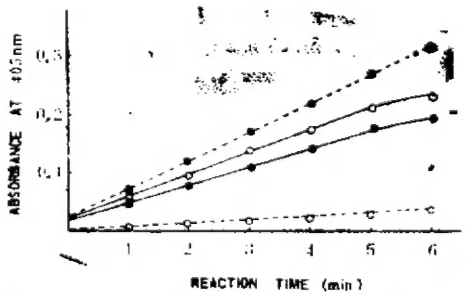

Fig. 7. The inhibitory effect of TMVFg on human thrombin in hydrolyzing substrate Cbz-Gly-Pro-Arg-PNA. Human thrombin control (●-----●), TMVFg control(○-----○), preincubated 0.2 ml human thrombin (10 U/ml) with 0.2 ml TMVFg(40 μg)at 37℃ for 10 min (○—○), 30 min (●—●).

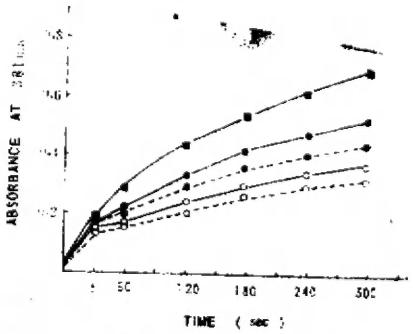

Fig. 8. Clotting of human fibrinogen by human thrombin after preincubating thrombin with TMVFg (80 μg) at 37℃ for various time intervals in 0.02*M* Tris buffer, pH 7.6. Control (■—■), preincubation time were 5 sec (●—●), 5 min (●-----●), 15 min (○—○), 30 min (○----○).

DISCUSSION

The fibrinogenase (TMVFg) coulb apparently prolong plasma thrombin time, plasma recalcification time and fibrinogen clot time. It also prolonged whole blood coagulation time *in vitro*. RATNOFF *et al*. (1976) have reported that the blood coagulation time was not prolonged if the remaining fibrinogen was above 20% of the original. After treatment of the human fibrinogen with TMVFg, two degraded products were found, one inhibiting, and the other enhancing the polymerization of fibrin.

Anti-thrombin action and anti-polymerizing effect of fibrinogen products degraded by β-fibrinogenase or by plasmin were also reported by (OUYANG *et al*. 1979, LARRIERU *et al*., 1975). TMVFg should be different from β-fibrinogenase (OUYANG *et al*., 1979) and plasmin as it degraded fibrinogen to produce FDP I which was capable of enhancing the fibrin polymerization.

TMVFg showed a very low activity toward Cbz-Gly-Pro-Arg-PNA which is a good substrate of thrombin. Thus TMVFg is not a thrombin like enzyme and could not form a fibrin clot with fibrinogen.

The results of our experiments suggested that the degraded products of fibrinogen have more functions than as reported. It is more likely that these products, *In vivo*, may act as regulating factors in blood coagulation system by positive feedback and negative feedback effects. The abnormal fibrinogen products degraded in some diseases may be responsible for the appearance of some symptoms of blood coagulation disorder such as disseminated intravascular coagulation (DIC).

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烙铁头蛇毒纤维蛋白原溶酶研究Ⅱ．对纤维蛋白原及凝血酶的作用

张　沄　熊郁良

(中国科学院昆明动物研究所)

摘　要

烙铁头（*T．mucrosquamatus*）蛇毒纤维蛋白原溶酶TMVFg能水解三肽底物Bz-Phe-Val-Arg-PNA，但对凝血酶的良好底物Cbz-Gly-Pro-Arg-PNA却活性甚低。TMVFg显著延长血浆凝血酶时间、血浆复钙时间及纤维蛋白原溶液凝血酶时间。同时，TMVFg体外也能延长全血凝固时间，表明其有抗凝作用。纤维蛋白原-纤维蛋白转换实验表明：TMVFg水解纤维蛋白原产生的纤维蛋白原断片（FDP）除具有抗凝血酶，抑制纤维蛋白聚合活性外，还能促进纤维蛋白的聚合。

进一步用FPLC分离TMVFg水解人纤维蛋白原混合液，得两个FDP断片功能峰，FDP组分Ⅰ和FDP组分Ⅱ。其中FDP组分Ⅰ能抑制纤维蛋白凝块形成；FDP组分Ⅱ能促进纤维蛋白凝块形成，抑制TMVA（烙铁头蛇毒血小板活化素，它可不通过ADP、花生四烯酸途径而诱导血小板聚集），但对ADP诱导的家兔血小板聚集无影响。TMVFg对凝血酶水解三肽底物Cbz-Gly-Pro-Arg-PNA及凝固纤维蛋白原的活性也有一定抑制作用。

实验证明，TMVFg抗凝的主要作用机理是其水解纤维蛋白原产生的断片对纤维蛋白原凝固的抑制作用、FDP断片抗凝血酶作用及TMVFg本身对凝血酶活性的抑制所引起的，但在二者之间，前者是主要的。

从研究结果发现：TMVFg水解纤维蛋白原所产生的断片有一类能加速凝血酶凝固纤维蛋白原的过程，这就发现了FDP断片的新功能。它证明了FDP断片作为血液凝固、纤溶正反馈调节因子的功能。这一类FDP断片还能抑制TMVA诱导的血小板聚集，因此，烙铁头蛇毒纤维蛋白原溶酶TMVFg将成为研究血液凝固调节系统及血小板聚集第三条途径的强有力试剂。

关键词：纤维蛋白原断片，纤维蛋白原，蛇毒，凝血酶